Human Junctional Epithelium: Demonstration of a New Marker, Its Growth In Vitro and Characterization by Lectin Reactivity and Keratin Expression

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We have studied lectin reactivity in normal human junctional, sulcular, and attached gingival epithelia with 15 lectins and identified the epithelia by parallel staining with monoclonal anti-keratin antibodies. *Dolichos biflorus* agglutinin reacted uniquely with junctional epithelium, not staining other gingival cells of non-blood group A_1 donors. We have demonstrated that the moiety recognized in junctional epithelium is not blood group A_1 antigen or Tn antigen. Using a panning technique with this lectin to isolate the cells, we have grown keratinocytes from human junctional epithelium, and compared their phenotype in vitro to that of cells grown from the sulcular and attached gingival epithelium. Colonies established from each epithelial type were examined in frozen section with the anti-keratin antibodies. All expressed keratin 14 (keratinocyte marker), keratins 4 and 13 (suprabasal non-cornification markers), and keratins 7, 18, and 19 (simple epithelia keratins). Keratins 1, 10, and 8 were not expressed. Vimentin, the intermediate filament of mesenchymal cells, was also expressed by all types of cells in culture. Thus we have shown that when cells from the three areas of the gingiva were grown in culture they revert to one phenotype, at least with respect to their keratin expression. These results support the hypothesis that the epithelial phenotype is influenced by the sub-epithelial mesenchyme, and it is this that is responsible for the unique phenotype of the junctional epithelium. *J Invest Dermatol 96:708–717, 1991*

The structure of human oral mucosa has marked regional variations; the epithelium consists of non-keratinized, orthokeratinized, and parakeratinized stratified squamous epithelia, as assessed by conventional histology, and there are also distinct morphologic differences in the lamina propria underlying these epithelia [1]. The gingival epithelium itself shows distinct differences: it can be divided into cornified, attached gingival epithelium, and the non-cornified, sulcular, and junctional epithelium (JE). JE and sulcular epithelium differ in both their morphology and origin. JE is unique because it is thought to originate from reduced enamel epithelium, with both an external and an internal basal lamina attaching it to the cementum of the tooth [2].

JE has elicited much interest due to its unique phenotype and structure, but also because of its role in the initiation of periodontal lesions. The loss of the JE attachment to the tooth is the primary indicator of destructive periodontitis. JE could be both a target for destruction, perhaps by pathogenic bacteria, or act as effector cells by producing enzymes such as gelatinase (type IV collagenase), which destroy basement membranes in the attachment loss.

Cytokeratins are the major structural proteins of all epithelial cells, comprising a group of 19 different polypeptides [3], the prod-

Manuscript received September 7, 1990; accepted for publication November 2, 1990.

This work was funded by the Medical Research Council.

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Abbreviations:

BSA: bovine serum albumin
BSL I: *Bandieraea simplicifolia* lectin
Con A: concanavalin agglutinin
DAB: diaminobenzidine tetrahydrochloride
DBA: *Dolichos biflorus* agglutinin
DMEM: Dulbecco's modified Eagle's medium
FCS: fetal calf serum
FITC: fluorescein isothiocyanate
Fuc: fucose
Gal: galactosamine
Glu: glucose
HBSS: Hanks' balanced salt solution
JE: junctional epithelium
LCA: *Lens culinaris* agglutinin
MoAb: monoclonal antibody
Man: mannose
NAcGal: N-acetyl-galactosamine
NAcGlu: N-acetyl-glucosamine
PBS: phosphate buffered saline
PHA-E: *Phaseolus vulgaris* erythro-agglutinin
PHA-L: *Phaseolus vulgaris* leuco-agglutinin
PNA: *peanut agglutinin*
PSA: *Pisum sativum* agglutinin
SBA: soybean agglutinin
SJA: *Sophora japonica* agglutinin
SSA: *Salvia sclarea* agglutinin
sWGA: succinylated WGA
RCA_{120}: *Ricinus communis* agglutinin 120
TBS: tris buffered saline
TRITC: tetramethyl rhodamine isothiocyanate
UEA I: *Ulex europaeus* agglutinin I
WGA: wheat germ agglutinin

0022-202X/91/$03.50

ucts of two distinct gene families [4]. Keratin expression in any epithelium is one of its most invariable characteristics, and the keratin combinations expressed reflect histologic type (simple, stratified), degree, and type of differentiation, and may also reflect functional state [5,6].

Lectins, carbohydrate binding proteins of non-immune origin, agglutinate cells and/or precipitate glycoconjugates. They have at least two sugar-binding sites, no enzymatic activity, and are of bacterial, animal, and plant origin [7]. They have been used to study glycoconjugate distribution in various epithelia [8–11] and for studying cellular differentiation and maturation [12–15]. Carbohydrate moieties, such as blood group antigens, have also been investigated in epithelia using monoclonal antibodies [15,16–18]. Blood-group antigen expression is related to cell maturity and differentiation pattern [15,19].

There have been few studies of lectin reactivity in the human gingiva [8,20], although some have described blood-group antigen expression in gingival epithelia using monoclonal antibodies. There has been, to our knowledge, one other study of human JE [8]. The mouse has been studied more extensively [21,22], showing that a number of ligands react specifically with the JE. Using a panel of monoclonal antibodies specific for single or small defined groups of keratins [23,24], it has been shown that the three human gingival epithelia each have a distinct expression pattern. JE was unique, expressing a number of keratins throughout all layers (unlike other areas of oral mucosa), such as keratin 14 and keratin 19. Recent evidence suggests that human JE also expresses a number of simple keratins [22].

By establishing in vitro assay systems, potential mechanisms of damage to JE by bacteria or inflammatory cells may be examined. It was, therefore, the aim of this work to grow JE keratinocytes in vitro and to establish whether the unique phenotype of JE was maintained in culture.

MATERIALS AND METHODS

Unless specified, tissue culture reagents were obtained from Gibco Ltd., Scotland, and all chemicals from Sigma, UK.

Tissue Sections Healthy gingivae attached to partially erupted third permanent molars from young adults or to other teeth extracted due to caries were dissected from the tooth and snap frozen. Six-micrometer frozen sections were cut, collected on gelatin-subbed slides, and stored at −70°C until stained. Gingivae from 12 donors were examined, including seven with JE.

Lectins Of the biotinylated lectins used, three reacted with mannose residues (Con A, PSA, LCA), one with fucose residues (UEA I), three with galactose residues (RCA^{120}, PNA, BSL I), two with N-acetyl glucosamine residues (WGA, succinylated WGA), four with N-acetyl galactosamine residues (SBA, DBA, SJA,SSA), and two with oligosaccharides (PHA-E, PHA-L).

Biotinylated lectins were from Vector Laboratories (UK). *Salvia sclarea* agglutinin (SSA) from Biosciences (Switzerland) was biotinylated using Biotin N hydroxysuccinimide ester (Vector) according to the manufacturer's specification. The success of biotinylation was assessed by dot-blotting: 10 μg/well of labeled or unlabeled SSA was adsorbed onto nitrocellulose paper for 1 h at room temperature, using a Biorad (UK) dot-blot apparatus. Non-specific binding was blocked by incubation with 2.5% BSA for 1 h at room temperature. Then a 1:300 dilution in phosphate buffered saline + 0.1% Tween 20 (PBS/Tween) of streptavidin-peroxidase complex (Amersham, U.K.) + 1% BSA was incubated for 30 min at room temperature. The blots were then incubated with 0.5 mg/ml diaminobenzidine tetrahydrochloride (DAB) in PBS + H_2O_2.

Antibodies Murine MoAb against keratin polypeptides were used to delineate the JE, sulcular, and attached gingival epithelia. The specific keratins recognized and the cells they stain in these epithelia are shown in Table I. We also used two MoAb (gifts from Dr. D. Anstee), BRIC 54 against blood-group A_1 antigen, and BRIC 111 against blood-group antigen Tn.

Specificities of Ligands The blood-group antigen specificities of DBA and SSA were checked by their ability to agglutinate blood group A_1 and blood-group O erythrocytes (London Hospital Blood Transfusion Unit, Ms. J. Sangster), and Tn-positive erythrocytes (gift from Dr. D. Anstee). Briefly, 15 μl of a 2% suspension of erythrocytes was mixed with an equal volume of 1.5 mg/ml DBA in PBS, 2 mg/ml SSA in PBS, or PBS only as a control, and mixed on a glass plate. Agglutination was assessed visually after 2 min.

The anti-A_1 and anti-Tn MoAb were tested for reactivity with erythrocyte membranes by immunoblotting. Erythrocyte ghosts were prepared by the method of Johnstone and Thorpe [25] using A_1, O, and Tn-positive erythrocytes. Briefly, 500 μl of packed erythrocytes were added to 20 ml of 5 mM phosphate buffer, and spun for 15 min at 20,000 × *g* at 4°C. After removing the supernatant, the centrifuge tube was tilted to allow the membrane to slide off the pellet, and the pellet was aspirated. Membranes were washed twice by centrifugation with phosphate buffer, resuspended in 50 μl of 10 mM tris buffer pH 8.2, and cooled on ice. Fifty μl of 1% sodium deoxycholate in Tris buffer was added, together with 1 μl of a 0.1 M solution of phenylmethylsulphonyl fluoride. For use in dot-blotting, this was made up to 2 ml with 1% sodium deoxycholate in Tris buffer pH 8.2, then diluted a further factor of either 10 or 50 in the same buffer. One hundred μl was added to each well of the dot-blot apparatus (approximately 5 μg or 1 μg of membrane protein per well) and incubated for 1 h. Wells were blocked with 2.5% BSA in PBS for 1 h, then incubated with undiluted anti-A_1 or anti-Tn antibody diluted 1:10 with PBS. It was then incubated with a 1:150 dilution of biotinylated sheep anti-mouse antibody (Amersham, UK) in PBS/Tween + 1% BSA for 1 h, then for a further 30 min with a 1:300 dilution in PBS/Tween + 1% BSA of streptavidin-peroxidase complex. Peroxidase activity was developed by incubation with DAB + H_2O_2 in PBS. Controls included substituting PBS + 1% BSA for either the biotinylated anti-mouse antibody or the streptavidin peroxidase complex.

Immunocytochemistry and Lectin Reactivity Tissue sections were acetone-fixed for 10 min at room temperature, air dried, and rehydrated in either PBS for antibodies or Hepes-buffered saline for lectins. All incubations were carried out at room temperature in a moist chamber, and all washes were for 2 × 5 min in Tris-buffered saline pH 7.4 (TBS).

Lectins were tested at 5 and 10 μg/ml in Hepes buffered saline, pH 7.2 (phosphate buffer was not used as it removes calcium, which some lectins require to express their activity). Tissue sections were incubated with the lectins for 1 h, then with a 1:150 dilution in TBS of streptavidin-peroxidase complex for 1 h. Peroxidase activity was exposed by incubation with DAB + H_2O_2 in TBS for 10 min, and the cells counterstained with Harris' hematoxylin.

The MoAb were applied undiluted to the tissue sections, except for M6B10 and MIC7, which were diluted 1:10 with PBS. All were incubated for 1 h. The sections were then incubated with a 1:150 dilution in PBS of biotinylated sheep anti-mouse antibody for 1 h. The streptavidin-peroxidase complex was added as described above, the peroxidase activity exposed with DAB, and the cells counterstained with hematoxylin. Controls included incubating lectins (10 μg/ml) for 30 min with their specific competitive sugars (BDH, U.K.) or an irrelevant sugar before incubation with the tissue sections. Con A, PSA, and LCA were incubated with 0.15 M mannose, UEA I with 0.15 M fucose, RCA^{120}, PNA, and BSL I with 0.15 M galactose, WGA and sWGA with 0.5 M N-acetyl glucosamine, SBA and SSA with 0.15 M N-acetyl galactosamine, DBA with 0.05 M N-acetyl galactosamine, SJA with 0.1 M N-acetyl galactosamine. PBS was substituted for the anti-keratin MoAb.

Preparation of Junctional Epithelium Cells Extracted teeth, without periodontal disease, had excess soft tissue removed, were washed twice in Hanks balanced salt solution (HBSS) containing 300 U/ml penicillin, 300 μg/ml streptomycin, and 5 μg/ml Fungizone, and a single-cell suspension of adherent cells was prepared

Table I. Monoclonal Antibodies Used, Keratins Recognized, and Tissue Specificities[a]

Antibody	Source	Reference	Keratins	Distribution in Gingiva
LP34	Dako	[45]	Many (5, 18, 6, 10, 14+)	All gingival epithelial cells
LHP1	Gift	[b]	1, 10	AE—suprabasal, SE
LHP3	Gift	[b]	10	negative, JE negative
M6B10	Bionuclear	[46]	4	AE—few scattered cells
M1C7	Bionuclear	[46]	13	SE—suprabasal, JE negative
LH8	Gift	[2]	14	AE & SE basal cells, JE all
M105	Bionuclear	[47]	7	
RPN1166	Amersham	[48]	8	Simple epithelia only
RPN1160	Amersham	[48]	18	(Merkel cells in gingiva)
M53	Bionuclear	[49]	18	
CAM5.2	Becton-Dickinson	[50]	7, 8, 18 ± 19	Weak SE basal, JE all cells (also simple epithelia)
RPN1165	Amersham	[51]	19	SE basal, JE all cells

[a] AE, attached epithelium; SE, sulcular epithelium; JE, junctional epithelium.
[b] Dr. I. Leigh (unpublished).

[26] with minor modifications. Briefly, the tooth was inverted and the crown incubated in HBSS containing 0.25% trypsin with 2 mg/ml dithiothreitol for 30 min at 37°C. It was then transferred to DMEM mixed with Ham's F12 medium in a 3:1 ratio, with 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone, and 15% FCS to inhibit further trypsin activity. Adherent cells were thoroughly scraped from the cemento-enamel junction with a scalpel blade and a single-cell suspension prepared by drawing the cell aggregates first through a 21 G needle then a 25 G needle. Cells were washed twice with HBSS.

Selection of Junctional Epithelium Cells by Panning DBA (Sigma Chemical Company Ltd.), 1 mg/ml in PBS, was incubated for 16–20 h at 4°C in 5-cm Petri dishes. Unadsorbed lectin was removed by washing with PBS, and the cell suspension added. After 1 h incubation at room temperature, non-adherent cells were washed off with PBS. Adherent cells were removed with a rubber policeman and the non-adherent cells were added back to the dish. After a 1-h incubation, this procedure was repeated, and the two adherent cell populations pooled. The concentration of lectin required for adsorption and the time required for cell adherence had previously been determined using A_1 erythrocytes and O erythrocytes as a negative control.

Immunocytochemistry on Panned Cells Cytospin preparations were made from cells initially isolated from the teeth and from adherent and non-adherent cells. They were fixed with acetone for 10 min. To show that cells were both DBA staining and stained for keratin 19, cytospin preparations from the mixed-cell population were rehydrated in PBS, incubated with anti-keratin 19 MoAb for 30 min at 37°C, washed with PBS, then incubated with a 1:20 dilution of anti-mouse FITC-conjugated MoAb (gift from Dr. M. Macey) in PBS for 30 min at 37°C and washed. Ten μg/ml rhodaminated DBA (Sigma) in PBS was added for 30 min at 37°C, and examined by fluorescence microscopy using appropriate FITC and TRITC filter combinations. To determine whether the adherent cells were A_1 positive, cytospin preparations were stained as described above with an anti-A_1 MoAb (gift from Dr. D. Anstee). Cell counts were made of DBA-positive and keratin 19-positive cells in both the adherent and non-adherent cytospin preparations. Controls involved substituting PBS for the primary MoAb pre-incubating the lectin for 30 min with 0.05 M N-Ac-galactosamine (competitive sugar), or pre-incubation with 0.15 M mannose (non-competitive sugar).

Isolation and Growth of Epithelial Cells The tissue remaining attached to extracted third permanent molars was dissected such that one portion contained the cornified attached gingival epithelium and the other the non-cornified sulcular epithelium. Each piece was washed twice in HBSS containing 300 U/ml penicillin, 300 μg/ml streptomycin, and 5 μg/ml fungizone, and incubated in HBSS containing 200 U/ml penicillin, 200 μg/ml streptomycin, 2.5 μg/ml fungizone, and 0.1% trypsin at 4°C for 16–20 h. The epithelial surfaces were scraped with a scalpel blade to release the cells, which were washed once in medium containing 15% FCS. Cells prepared in this way, and the adherent population of cells prepared as described above were added to 5-cm petri dishes (Cel-Cult, Sterilin), together with 3×10^5 mitomycin C-treated Swiss 3T3 cells as a feeder layer [27]. The cells from the three sites were grown under the same conditions in DMEM mixed with Ham's F12 medium in a 3:1 ratio, with 15% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone, 0.4 μg/ml hydrocortisone (Sigma), and 5 ng/ml cholera toxin (Sigma). Epithelial cell growth factor (Sigma) was added at 20 ng/ml after 3 d. The cells were grown in a humidified incubator at 37°C in an atmosphere of 5% CO_2 in air. Two dishes of cells from the adherent population were maintained in culture, one for 24 h and the other for 48 h. At these time points the cells were removed from the dish with a rubber policeman, cytospin preparations made, and the cells examined for their DBA reactivity as described in the next section. All other cells were allowed to grow with the medium being changed twice weekly. Contaminating fibroblasts were removed by incubating the cells with 0.02% ethylenediamine tetra-acetic acid.

Where more than one colony of epithelial cells had become established, one colony in each dish was selected and isolated using a sterile rubber ring. All adjacent colonies were removed by trypsinization and the cells transferred to a separate petri dish. The colonies were allowed to grow until they were approximately 3 cm in diameter (18–24 d), and were then removed from the dish by incubation with 1.25 U/ml dispase (Boehringer Mannheim, FRG) in DMEM at 37°C and snap frozen. Six-μm transverse frozen sections were cut and collected on gelatin-subbed slides and stored at −70°C until stained. Colonies originating from buccal gingival epithelium, from sulcular epithelium, and from the enriched population of JE were examined.

Immunocytochemistry on Colony Sections Sections were either left unfixed or were acetone fixed for 10 min at room temperature. They were then stained as described above with the MoAb listed in Table I, with anti-vimentin antibody (Dakopatts. Denmark) and with biotinylated DBA.

RESULTS

Specificities of Blood Group A_1 and Tn-Specific Lectins DBA agglutinated both the Tn- and the A_1-positive erythrocytes, whereas SSA agglutinated only Tn-positive erythrocytes, demonstrating its lack of reactivity with the GalNAc residue in the A_1 saccharide residues. Neither lectin agglutinated group-O erythrocytes.

Specificities of Antibodies for Blood Group A_1 and Tn Both antibodies reacted only with erythrocytes bearing their specific antigen. Neither reacted with group-O erythrocyte membranes.

Immunocytochemistry and Lectin Reactivity The staining pattern of the anti-keratin MoAb in human gingiva has been de-

scribed elsewhere [23], and were used in this study only to distinguish the three types of epithelia, particularly the JE. Briefly, LHP1 and LHP3 stained suprabasal cells of the attached gingival epithelium; M6B10 and MIC7 stained suprabasal cells of the sulcular epithelium; RPN1165 stained basal cells of sulcular epithelium, Merkel cells and all JE cells.

The lectin staining pattern in the gingiva is summarized in Table II.

1. Reactive with Mannose Residues: None have any blood-group specificities, and show a variety of staining patterns within the gingiva. Con A reacted with the membranes of epithelial cells of the higher spinous and granular layers, weakly with basal cells and not with the cornified layer. There was some intracellular staining in all cells, particularly of the JE. *Pisum sativum* agglutinin (PSA) reacted with the membranes of all epithelial cells except those of the cornified layer, and also with the cytoplasm of JE cells. *Lens culinaris* agglutinin (LCA) gave only very faint membrane staining with all epithelial cells.

2. Reactive with Fucose Residues: UEA I stained the cytoplasm of JE cells and the membranes of the epithelial cells, faintly in the basal cells and within the rete pegs but more strongly toward the granular layer, especially in attached gingival epithelium (see Fig 1*a*).

3. Reactive with Galactose Residues: RCA^{120} stained the membranes of all epithelial cells fairly weakly in the basal layers and more strongly towards the granular layers; the cornified layer was unstained. The JE cells were also stained intracellularly. PNA stained weakly the basal cells of the attached gingiva and both the membrane and cytoplasm, with the membranes of the suprabasal cells staining more strongly. In sulcular epithelium, basal cells stained more strongly than the suprabasal cells. JE showed strong intracellular and membrane staining (see Fig 1*b*). BSL I did not stain basal cells or JE and showed a variable staining pattern with the other epithelial cells, staining the cell membranes in the granular layer of attached gingival epithelium, and staining irregularly in the higher layers of sulcular epithelium.

4. Reactive with NAcGlu Residues: Wheat germ agglutinin (WGA) stained the membranes of all cells in all layers of the epithelium, and also the cytoplasm of JE. Succinylated WGA (sWGA) did not react with basal cells of the epithelium but stained the membranes of suprabasal cells of all layers, more strongly in the granular layers. JE was also stained.

5. Reactive with Oligosaccharides: *Phaseolus vulgaris* erythro-agglutinin (PHA-E) stained basal cells of the attached gingival and sulcular epithelium weakly and the membranes of suprabasal cells more strongly, particularly those of the attached gingival epithelium. JE was positive through all its layers. *Phaseolus vulgaris* leuco-agglutinin (PHA-L) stained the membranes of all the epithelial cells. JE cells stained strongly intracellularly (see Fig 1*c*).

6. Reactive with NAcGal Residues: Soybean agglutinin (SBA) reacted with the epithelium. Basal cells at the base of the rete pegs were unstained with the membranes of all suprabasal cells stained except the cornified layer. JE cells showed both membrane and cytoplasmic staining (Fig 1*d*). This pattern occurred irrespective of whether the donor was blood group A_1 or not (determined by the staining pattern of A_1 antibody). *Sophora japonica* agglutinin (SJA) did not stain the epithelium except in A_1-positive donors, where there was faint staining on the membrane of upper suprabasal cells of the attached gingival epithelium. SSA did not react with anything in the gingiva. In samples from A_1-positive donors, *Dolichos biflorus* agglutinin (DBA) reacted with endothelial cells, the membranes of suprabasal cells of the attached gingival epithelium and a few cells in the high suprabasal layers in sulcular epithelium. This pattern of reactivity was the same as that obtained with anti-A_1 antibody. All these areas were unstained in samples from non-A_1 donors. However, JE from both A_1-positive and -negative donors showed strong intracellular and membrane staining with this lectin. This is shown in Figs 2*a–c*, where JE is delineated by reactivity with a MoAb recognizing keratin 19, and an adjacent section stained with DBA, where a clear cut-off is seen at the boundaries of the JE and sulcular epithelia.

Anti-Tn antibody did not react with the gingiva. Anti-A_1 antibody reacted with endothelial cells and suprabasal cells of attached gingival and sulcular epithelia. JE was unstained.

Immunocytochemistry on Panned Junctional Epithelial Cells Cells removed from the teeth were approximately 30% DBA positive. When examined by fluorescence microscopy for the DBA binding and keratin 19, at least 60% of cells consistently fluoresced at both wavelengths showing that they were both DBA and keratin 19 positive. The majority of the remaining cells were positive only for keratin 19. Gingival sections stained with anti-keratin 19 show that keratin 19 is expressed by all cells of the JE and by basal cells of the sulcular epithelium. It is therefore likely that the keratin 19-positive/DBA-negative cells are basal cells from any residual sulcular epithelium adherent to the tooth, whereas those staining with both labels were from the JE. None of the adherent cells stained for A_1 antigen, probably because cells expressing this antigen are the higher suprabasal cells in the gingival and sulcular epithelium [15] and such tissue was removed before the digestion procedure.

Table II. Summary of Lectin Reactivities in the Gingiva[a]

	Attached Gingival Epithelium			Sulcular Epithelium		Junctional Epithelium	Connective Tissue	BM
	B	SB	C	B	SB			
Con A	(+)	+	−	(+)	+	+	All stained	+
SBA	−	+	−	−	+	+	Endothelium +	(+)
WGA	+	+	−	+	+	+	All stained	−
DBA	−	+[b]	−	−	±[b]	+	Endothelium +[b]	−
UEA I	(+)	+	−	(+)	+	+	Endothelium +	−
RCA^{120}	+	+	−	+	+	+	All stained	+
PNA	(+)	+	−	+	(+)	+	All stained	+
BSL I	−	+[b]	(+)	−	±[b]	−	Endothelium +[b]	(+)
PSA	+	+	−	+	+	+	All stained	+
LCA	(+)	(+)	−	(+)	(+)	(+)	All stained	+
PHA-E	(+)	+	−	(+)	+	+	All stained	+
PHA-L	+	+	−	+	+	+	Endothelium	+
SJA	−	(+)[b]	−	−	−	−	Endothelium +[b]	−
sWGA	−	+	−	−	+	+	Unstained	−
SSA	−	−	−	−	−	−	Unstained	−

[a] ±, some cells positive, some negative; (+), faint staining; −, unstained; B, basal cells; SB, suprabasal cells; C, cornified layer; BM, basement membrane.
[b] Blood group dependent.

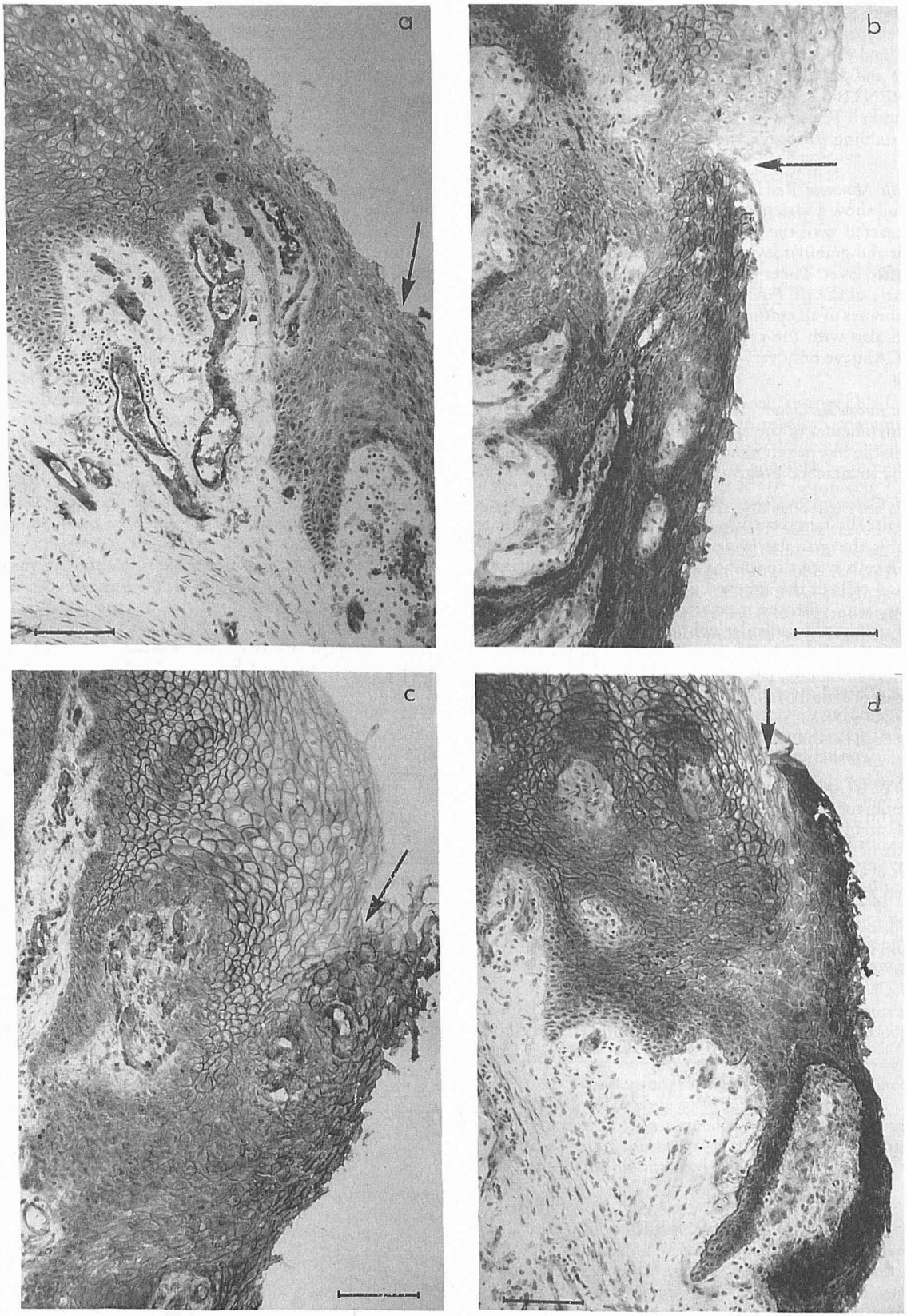
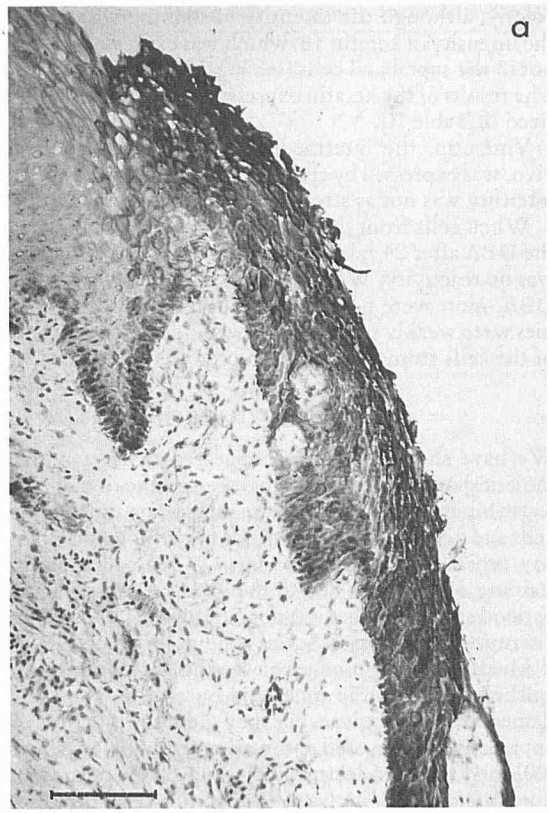
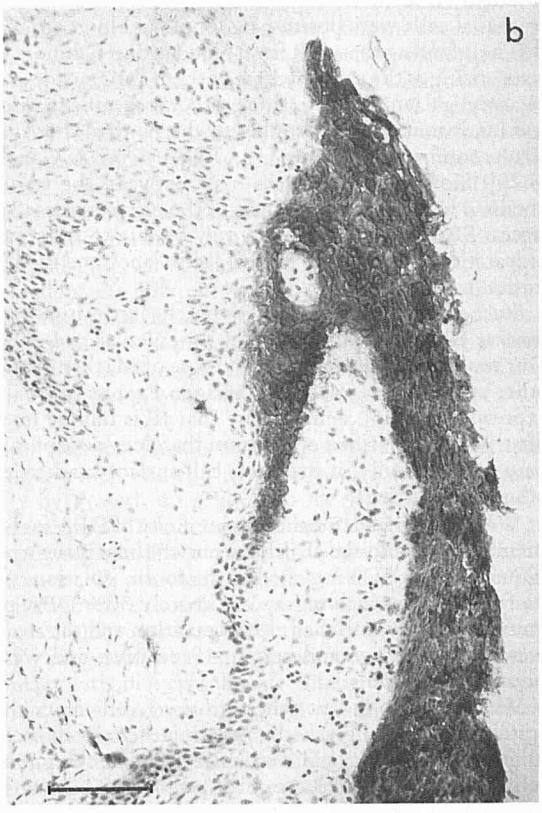
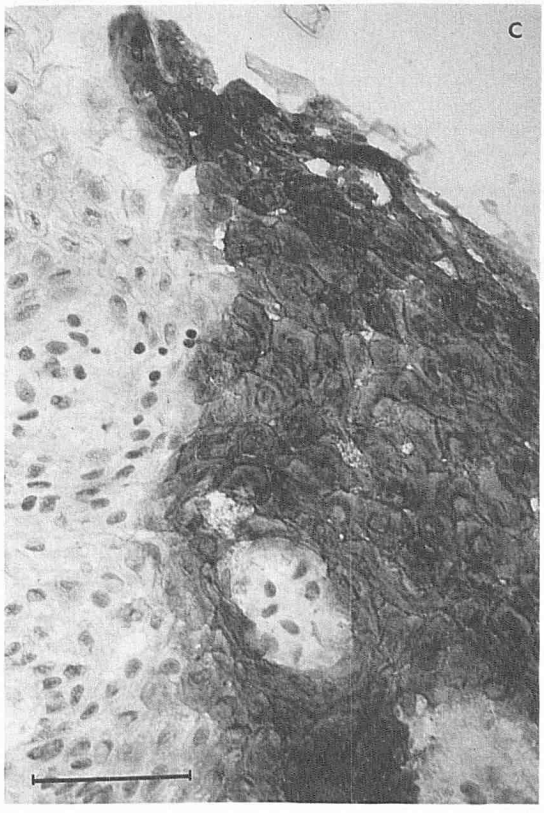


Figure 1. Sections showing the boundaries of the junctional and sulcular epithelia *(arrows)*. The photographs are oriented so that the junctional epithelium is at the bottom and the sulcular epithelium at the top. All, magnification ×200. *Bar,* 50 μm. *a,* Stained with UEA I. There is no clear distinction between the junctional and sulcular epithelia. The membrane staining is stronger higher in the suprabasal cells of the sulcular epithelia. The endothelial cells are strongly positive. *b,* Stained with PNA. The junctional epithelium is stained strongly both intracellularly and on the membrane. The staining on the membranes of the cells in the sulcular epithelium is weaker. *c,* Stained with PHA-L. There is strong intracellular staining in the junctional epithelium and the basal cells of the sulcular epithelium. Only the membranes of the suprabasal cells of the sulcular epithelium are stained. *d,* Stained with PNA. There is strong intracellular staining in the junctional epithelium. The basal cells of the sulcular epithelium are unstained. The membranes and cytoplasm of the suprabasal cells of the sulcular epithelium are stained.







When frozen sections from five colonies grown from the adherent cell population were examined for their keratin expression and DBA reactivity, all were found to have an identical phenotype to those grown from the other areas of the gingiva (see below). Thus, given that 40% of panned cells were DBA negative, the probability that all these colonies will have originated from non-JE cells is: $0.4^5 = 0.01024$.

Immunocytochemistry on Colony Sections Morphologically, the colonies showed stratification, comprising two to three layers of cells. Colony sections were oriented so that the "basal" cells were those that had been in contact with the dish, and typically these were small, regularly shaped cuboidal cells. The suprabasal cells were more elongated and flattened with a greater variety of sizes. To show that cells in the colonies were epithelial in origin, LP34, a panepithelial marker, was used. This recognizes many keratins and therefore stains both simple and stratified epithelia.

Whatever their site of origin, all colonies had the same keratin profile in culture. Keratins 1 and 10, suprabasal cornification markers, were not expressed by any cells in vitro. However, keratins 4 and 13, suprabasal non-cornification markers, found in sulcular epithelium, were expressed by the suprabasal cells in vitro (see Fig 3*a*), showing that there was some differentiation in these cultures. Basal cell marker LH8, positive in basal cells in vivo, was positive in all suprabasal cells and some basal cells in vitro. Keratin 19 was expressed in vivo by simple epithelia, Merkel cells, by the basal cells of the sulcular epithelium and all JE cells. In vitro this keratin was expressed by all epithelial cells (see Fig 3*b*). Keratins 7, 8, and 18 were not detected in the epithelia of the gingiva. In vitro, however, keratin 7 was expressed irregularly by cells throughout the whole

Figure 2. Sections showing the boundaries of the junctional and sulcular epithelia. The photographs are oriented so that the junctional epithelium is at the bottom and the sulcular epithelium at the top. *a*, Stained with antibody RNP 1165, which recognizes keratin 19. The basal cells of the sulcular epithelium and all cells of the junctional epithelium are stained. Magnification ×200. *Bar,* 50 μm. *b*, Stained with DBA. All the junctional epithelial cells are stained. Magnification ×200. *Bar,* 50 μm. *c*, Stained with DBA. Higher magnification (×400) showing the membrane and cytoplasmic staining of the junctional epithelial cells, at the boundaries of the junctional and sulcular epithelia. *Bar,* 50 μm.

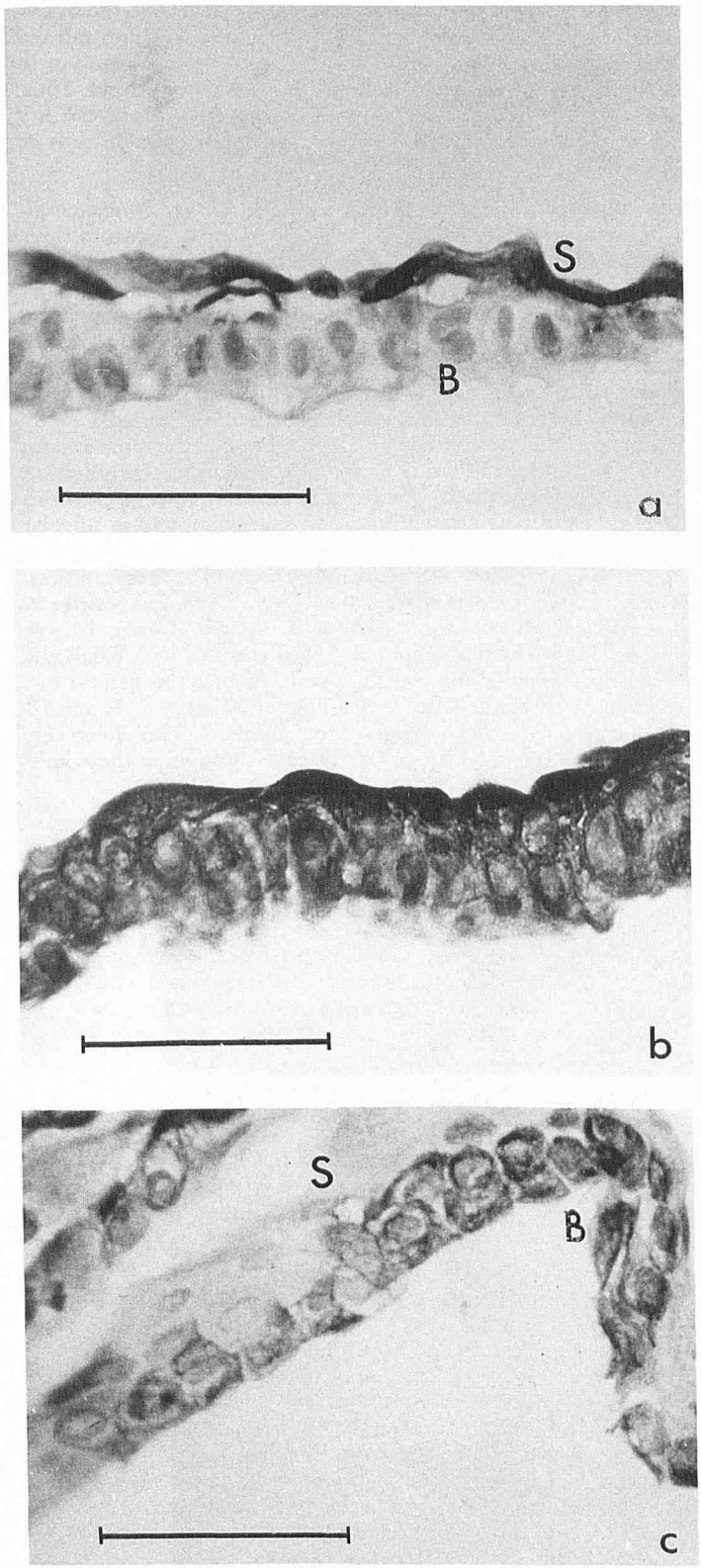


Figure 3. Frozen sections of colonies stained with anti-keratin antibodies. All magnification ×650. *Bar,* 50 μm. *a,* Colony section stained with M6B10, a suprabasal non-cornification marker, recognizing keratin 4. The suprabasal cells (S) are positive with this antibody, whereas the basal cells (B) are negative. *b,* Colony section stained with RPN 1165, recognizing keratin 19. All cells are positive with this antibody. *c,* Colony section stained with RPN 1160, a simple epithelial marker, recognizing keratin 18. The basal cells (B) are positive with this antibody, whereas the suprabasal cells (S) are negative.

colony, although the intensity of staining was fairly weak, as was the intensity of keratin 18, which was expressed in the basal cells but not in the suprabasal cells (see Fig 3*c*). Keratin 8 was not expressed. The results of the keratin expression in vivo and in vitro are summarized in Table III.

Vimentin, the intermediate filament of mesenchymal cells in vivo, was expressed by epithelial cells in vitro, although the staining intensity was not as strong as the anti-keratin antibodies.

When cells from the adherent cell population were stained with the DBA after 24 h in culture, reactivity was faint. After 48 h there was no reactivity. When sections of colonies were stained with the DBA, most were negative although suprabasal cells in some colonies were weakly stained. This was independent of the site of origin of the cells from which the colony was established.

DISCUSSION

We have shown that DBA appeared to react with a moiety (or moieties) on and within JE that are specific to that epithelium. The lectin binds strongly to both the membrane and the cytoplasm of the cells and not, provided that the donor is blood group A_1 negative, to any other gingival components. We also demonstrated that the staining of JE is not due to the group A_1 antigen and, using both antibody and SSA, that it is not Tn antigen [28], a cryptantigen with a terminal, α-linked NAcGal residue to which DBA also binds.

Murase et al [8] used seven lectins, including DBA, to compare epithelial cells of the inner and outer gingiva of healthy and inflamed human gingivae, but they did not show that JE had a different reactivity compared to the other epithelia. Hormia and Virtanen [20] used fourteen lectins, including DBA, to examine samples of human marginal gingiva, although not JE, where the blood group status of the donor was known. They reported a similar distribution to our own with DBA in attached gingival epithelium, but the epithelial cells were positive in donors of blood groups A, AB, and B. Their finding that cells from blood group B donors were positive is surprising as the carbohydrate of the B antigen does not contain an N-acetyl galactosamine residue. DBA is specific for blood group A, and binds much more strongly to the A_1 than the A_2 moiety. Our results confirm those using UEA I, RCA^{120}, SJA, Con A, and WGA [8,20]. However, differences exist between the staining patterns obtained in our study and those of the other studies [8,20] with the lectins SBA and PNA. These may be due to differences in tissue preparation, fixation, the type of lectin label, and the lectin concentration used.

Mackenzie [21] demonstrated that the lectin-binding reactivity of murine JE differed from that of the sulcular and oral epithelium. Our results with the lectins, and those of Mackenzie [21], as well as other studies of blood-group antigen expression [15] and keratin expression [22,23], demonstrate that JE is unique in two respects. Firstly, it is a stratified epithelium that does not appear to differentiate, and secondly, it expresses cell surface markers not found in other oral epithelia.

We have utilized the unique specificity of DBA for binding to the membranes of human JE cells to enrich them using a panning technique. Because JE is attached to the tooth, isolation of the cells is a useful first step, yielding approximately 30% DBA-positive cells. Panning further enriched this population and the majority of cells were DBA positive and keratin 19 positive, and were, therefore, presumably JE cells.

We can be confident when growing cells from the two other epithelia of the gingiva—by isolating them by dissection—of the origin of these cells, and consequently relate the phenotype of the cells grown in vitro to those of the parent tissue. As we have demonstrated, cells grown from the DBA-enriched JE have the same phenotype, at least for keratin expression and DBA reactivity, as those from the other areas of the gingiva. As it is unlikely ($p = 0.01024$) that all the colonies we examined originated from cells from the other two gingival epithelia, we have thus demonstrated that it is very likely that keratinocytes grown from JE have adopted the same

Table III. Staining Pattern of Keratinocytes In Vitro Compared to That In Vivo[a]

Keratin Number	1–19	1,10	14[b]	4,13	7	8	18	19	Vimentin
Cultured Keratinocytes									
Basal	+	−	±	−	±	−	+	+	+
Suprabasal	+	−	+	+	±	−	−	+	+
Tissue Sections									
Buccal epithelium									
Basal	+	−	+	−	−	−	−	−	−
Suprabasal	+	+	−	−	−	−	−	−	−
Sulcular epithelium									
Basal	+	−	+	−	−	−	−	+	−
Suprabasal	+	−	−	+	−	−	−	−	−
Junctional epithelium									
All layers	+	−	+	−	−	−	−	+	−

[a] ±, irregular staining; −, no staining; +, staining.
[b] Antibody LH8 staining (restricted keratin 14).

phenotype as those grown from the other areas of the gingiva. We have had to assume that the plating efficiency of each cell type was equivalent. Our observation that, after 24 h in culture, the staining of a population of previously mainly DBA-positive cells was faint and was absent after 48 h would indicate that it is not that the plating efficiency of JE cells is low compared to the other keratinocytes in the enriched population, and are therefore overgrown, but that the DBA-binding moiety is a unique feature of the cells only in situ, and the control mechanisms for influencing it are lost once the cells have been isolated.

There has only been one other attempt, as far as we are aware, to grow JE in vitro. Altman, Nelson, Povolny et al [29] have grown cells from rat JE, palatal and gingival epithelium by three different methods. The cells were characterized for keratin expression in vivo and in vitro. When cells from the JE and gingival epithelium were grown on an artificial basement membrane, keratin expression in vitro and in vivo were the same. The JE was reactive with antibodies recognizing desmoplakin I and II and non-reactive with an antibody recognizing keratins with molecular weights of 56.5 (keratin 10) and 65–67 KDa (keratins 1 and 2), the suprabasal cornification markers. In contrast, those from gingival epithelium showed the opposite staining pattern. Our own results do not confirm this observation, but one reason may be that the cells in our study were not grown on an artificial matrix. Altman et al [29] also demonstrated that when JE and gingival epithelial cells were grown by other methods, they adopted a different but identical phenotype in vitro, and that the expression of certain keratin polypeptides depended on whether the cultures were confluent. They also observed that none of the epithelial cells in vitro reacted with an antibody recognizing vimentin, in contrast to our own data and those of a number of other studies [30–34].

A number of attempts have been made previously to adapt the conditions under which oral epithelial cells were grown or maintained, mainly by growth on a filter, to see whether they could adopt the phenotype of JE [35,36]. The latter study found that an antibody recognizing a keratin with the distribution in vivo of keratin 19 reacted with cells growing along a filter. However, as we and others [34] have shown, this may be because epithelial cells in culture express keratin 19, regardless of the tissue of origin rather than, as Salonen [36] suggested, that this expression was due solely to growth in contact with an inert substrate. The other unique feature of normal JE, i.e., that it is non-migrating, was also not reproduced in this model. Mackenzie [21] has described an in vivo model in the mouse that seems to reproduce many of the characteristics of JE. In this model, epithelia from different areas of the mouse oral cavity were transplanted onto areas of deep connective tissue. In this environment the epithelium is non-migrating and adopts a new phenotype, showing the same pattern of reactivity for both lectins and keratins as the JE in situ.

Our results are consistent with the hypothesis that the phenotype of JE, as well as all other epithelia, is influenced by the sub-epithelial connective tissue [37,38], and that this was the reason that the DBA reactivity of the JE was lost after 48 h in culture. Although some cells in some colonies were positive for DBA, the staining pattern was independent of the site from which the cells were obtained. As only the suprabasal cells were stained, it is likely that this was due to the expression of blood group A_1 antigen [39,40]. Breitkreutz, Bohnert, Herzmann et al [41] demonstrated that mouse skin keratinocytes cultured for 48 h lose their expression of keratins with molecular weights 67 and 58 KDa, which was paralleled by an increase in the expression of a number of keratins unique to these cells when grown in culture. This group also demonstrated that if the cultured epithelial cells were transplanted back to the original site in the mouse, the changes in keratin expression that had occurred in vitro were reversible and the epithelium regained its original phenotype.

Van Muijen, Warnaar and Ponec [34] using human skin keratinocytes and culture conditions comparable to those in our study, found—as have we and others [42]—that cultured keratinocytes expressed different combinations of keratin polypeptides. Skin keratinocytes expressed keratins 4, 13, and 19 and occasionally 10 in their superficial cells, vimentin in the majority of basal cells, and did not express keratins 8 and 18 in any cell. This profile differs from the keratins expressed by human gingival keratinocytes in vitro where, although keratins 4 and 13 are expressed suprabasally, we have shown that keratin 19 and vimentin were expressed by all cells. In addition, although keratin 8 was not detected, keratin 18 was expressed by the basal cells. The differences in vitro may not be explained by differences in the specificities of the monoclonal antibodies used, as most of the antibodies used in our study and that of Van Muijen et al [34] were from the same clones. It may be that small differences in the culture medium may be responsible for the differences in expression. It has been shown that high retinol concentrations will induce keratin 19 [43], and Southgate, Williams, Trejdosiewicz et al [33] demonstrated that cholera toxin and epithelial cell growth factor modulated the differentiation of human oral epithelial cells in culture. It should also be recognized that certain keratin antigens may be masked in situ and are revealed by biochemical methods, as has been shown by Woodcock-Mitchell, Eichner, Nelson et al [44], which could account for differences observed by different groups of workers. It may also be that there is a fundamental difference in keratin expression between keratinocytes from two different sites and that some control of keratin expression may be preserved in the cultured cells.

In summary, we have shown that the DBA has a unique specificity for human JE, not reacting with any other components in the gingiva of non-blood group A_1 donors, and that this reaction is not with A_1 or Tn antigens. The identification of a unique marker for human JE has many potential uses. In this study we have used DBA binding to select and grow human JE in culture. It is also possible that this property could be exploited for use as a measure of loss of JE attachment to the tooth or damage to JE cells, thus having potential uses in assessing disease activity or pathogenic mechanisms in periodontal diseases. We have also shown that keratinocytes from JE can

be grown in vitro. The cells appear to adopt the same phenotype as keratinocytes grown from the other areas of the gingiva. These results support the hypothesis that the unique phenotype of JE is due to directive or permissive influences from the underlying connective tissue, and that removal from these influences leads to a rapid loss of this phenotype.

We would like to thank Dr. J. Sterne for statistical advice, Dr. I. M. Leigh for the gift of the LH8, LHP1, and LHP3 antibodies, Dr. D. Anstee for the anti-blood group A_1 and anti-Tn antibodies, and the Tn erythrocytes, Ms. J. Sangster for the blood group O and A_1 erythrocytes, and Dr. M. Macey for the anti-mouse FITC-conjugated MoAb.

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ANNOUNCEMENT OF AWARDS

The KAO Corporation of Japan has in 1990 established the Thomas B. Fitzpatrick Research Awards, A New Research Career Development Program in Dermatology, an annual award of $50,000 yearly for post-doctoral Ph.D. basic scientists or physicians who are destined for a research career in investigative dermatology with special emphasis on basic science. The rationals of this award is to bridge the critical gap following the completion of training and before there is stable support.

Awardees for the funding year July, 1991–June, 1992 have been selected by a Scientific Committee. Many factors are considered in the final selection of the Fitzpatrick awardee, among them age, scientific milieu, originality of research, quality of previous training, and evidence of potential for a career in research on the biology of skin.

The awardees are Ponciano D. Cruz, Jr., M.D., University of Texas Southwestern Medical Center and James Glenn Krueger, M.D., The Rockefeller University. Dr. Cruz is currently an Assistant Professor in the University of Texas, Department of Dermatology, working in the area of Photoimmunology. Dr. Krueger is an Assistant Professor in the Laboratory for Investigative Dermatology at Rockefeller University, working in cell biology and biochemistry.

This is a three-year program, with additional awards to be made for 1992–1993 and 1993–1994. Interested applicants are encouraged to apply. Application forms are available from KAO Scientific Committee, c/o Management Consultants, 395B Essex Street (#306B), Beverly, MA 01915. Application deadline is October; Announcement of awards is in January; Funding will be available in July.